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(71) Applicant: BAYLOR COLLEGE OF MEDICINE [US/US]; One Baylor Plaza, Houston, TX 77030–3498 (US).

(72) Inventors: DAVID, Ronald, L.; 5207 Paisley Lane, Houston, TX 77096 (US). ZHU, Xin–Ran; Institute for Neuronale Signal Transduction, Center for Molecular Neurobiology, Martinistrasse 85/Falkenried 94, D–20246 Hamburg (DE). GROTEWIEL, Michael, S.; 5935 Spellman, Houston, TX 77096 (US). BECK, Christine, D., O.; Apartment No. 4, 7218 Cambridge, Houston, TX 77030 (US). WU, Kwok–Hang; Columbia University, Dept. of Biological Sciences, New York, NY 10027 (US).

(74) Agent: DAVIDSON, Ross, E.; Fulbright & Jaworski, Suite 5100, 1301 McKinney, Houston, TX 77010–3095 (US).

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(54) Title: METHOD OF SCREENING FOR NEUROPHARMACEUTICALS USING THE *DROSOPHILA* GENE *VOLADO* AND ITS MUTANTS

(57) Abstract

A new memory factor *Volado* is described along with the corresponding gene, protein sequences, and two mutants identified as Vol^1 and Vol^2. The locus encodes two isoforms of a novel α–integrin expressed preferentially in mushroom body cells. *Volado* mutants display an impairment of olfactory memories within 3 minutes after training, indicating an essential role for the integrin in short–term memory processes. Conditional expression of a Volado transgene during adulthood rescues the memory impairment. This rescue of memory is reversible, fading over time along with expression of the transgene. The present invention provides a novel method for screening for cognitive enhancers using the *volado* and integrin proteins. This procedure involves inserting a gene sequence and coding for a *volado* or integrin protein into test cells in culture under conditions where said gene sequence expresses the *volado* or integrin protein in said test cell, adding a test compound to the cell culture or cell homogenate, and measuring the effect of test compound and the activity of the *volado* or integrin proteins. This screening procedure can also be used in organisms such as Drosophila flies.

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METHOD OF SCREENING FOR NEUROPHARMACEUTICALS USING THE *DROSOPHILA* GENE *VOLADO* AND ITS MUTANTS

The work herein was supported by grants from the United States Government. The United States Government may have certain rights in this invention.

Field of the Invention

The present invention relates generally to the field of screening for pharmacological agents and drugs which can be used as cognitive enhancers. More specifically, it relates to the field of screening for modulators of integrin function as a screen for cognitive enhancers.

Background of the Invention

The ability to acquire and process information about the environment (learning) and to store and retrieve this information over time (memory) is fundamental for many organisms. Learning and memory are expressed as modifications of animal behavior (conditioning) which emerge from the function of molecules within neurons, the integrated action of many neurons comprising neural circuits, and from the engagement of multiple circuits.

Two broad phases of memory have been distinguished from behavioral and cellular studies: short-term memory and long-term memory. Short-term memory, which lasts from minutes to hours, is thought to occur through changes in synaptic efficacy produced by rapid and transient biochemical alterations in the relevant neurons. Byrne, J.H. *et al.*, in *Advances in Second Messenger and Phosphoprotein Research* Shenolikar, S. & Nairn, A.C. (eds.) 47-107 (1993); Chetkovich, D.M. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:6467-6471 (1991); Ghirardi, M. *et al.*, *Neuron* 9:479-489 (1992); Davis, R. L., *Physiological Reviews* 76:299-317 (1996);

Hawkins, R.D. *et al.*, *Annual Rev. Neurosci.* 16:625-665 (1993). In contrast, long-term memory, which lasts from days to years, is thought to occur through changes in synaptic efficacy produced by the restructuring of synapses due to altered gene expression. Davis, H.P. & Squire, L.R., *Psychol. Bull.* 96:518-559 (1984); Montarolo, P.G. *et al.*, *Science* 234:1249-1254 (1986); Tully, T. *et al.*, *Cell* 79:35-47 (1994); Schacher, S. *et al.*, *Science* 240:1667-1669 (1988); Bailey, C.H. & Kandel, E.R., *Annual Rev. Physiol.* 55, 397-426 (1993). The formation of long-term memory, but not short-term memory, has therefore been thought to rely upon morphological restructuring of synapses using mechanisms similar to those used for brain development.

In *Drosophila*, the formation of olfactory memories is scripted in cyclic AMP (cAMP) signaling in neurons of the mushroom bodies. Davis, R.L., *Physiological Reviews* 76:299-317 (1996); Davis, R.L., *Neuron* 11:1-14 (1993); Davis, R.L. & Han, K.-A., *Current Biology* 6:146-148 (1996). A significant series of studies linking cAMP signaling, mushroom bodies, and olfactory learning demonstrated that three genes required for normal learning - *dunce* (*dnc*), *rutabaga* (*rut*), and *DCO* (the genes for cAMP phosphodiesterase, adenylyl cyclase, and the catalytic subunit of protein kinase A (PKA), respectively) - are all expressed preferentially in mushroom bodies. Davis, R.L., *Neuron* 11, 1-14 (1993). Moreover, the characterization of two other learning genes of *Drosophila* is consistent with a dominant role for cAMP in modulating the physiology of neurons that mediate behavioral plasticity. The *amnesiac* gene encodes a peptide similar to PACAP (pituitary adenylyl cyclase activating peptide) Feany, M. S. & Quinn, W.G., *Science* 268, 869-873 (1995) and dCREB2 encodes a transcription factor that may mediate cAMP-dependent gene expression. Yin, J.C.P. *et al.*, *Cell* 79:49-58 (1994). A leading hypothesis that has emerged from these studies is that mushroom bodies function as the integration and memory center for olfactory learning by employing the cAMP signaling system. Davis, R.L., *Physiological Reviews* 76:299-317 (1996); Davis, R.L., *Neuron* 11:1-14 (1993).

Mushroom bodies are bilateral clusters of about 2500 neurons situated in the dorsal and posterior cortex of each brain lobe. Davis, R.L. & Han, K.-A., (1996). *Current Biology* 6:146-148 (1996). These cells extend dendrites into a neuropil (calyces) just ventral to the cell bodies where inputs arrive from the antennal lobes and other centers conveying sensory information. The axons of mushroom body cells fasciculate to form the peduncle that projects anteriorly to the anterior of the brain. There it bifurcates, with some processes extending medially to comprise the neuropil region known as the β and γ lobes, and others extending dorsally to comprise the α lobe Strausfeld, N.J., *Atlas of an Insect Brain* (1976). Although the mushroom bodies receive inputs from many sensory modalities through the calyces and lobes and are required for olfactory learning, they are not required for olfaction per se (Heisenberg, M. *et al.*, *Neurogenetics* 2:1-30 (1985); Menzel, R. *et al. The Behavior and Physiology of Bees (L.J. Goodman & R.C. Fisher eds.)* (1991); DeBelle, S.J. & Heisenberg, M., *Science* 263:692-695 (1994).

Despite the coherent evidence pointing to the cAMP signaling system, many different types of molecules must be engaged during learning to effect the overall physiological changes in the relevant neurons. Indeed, an assortment of protein kinases, transcription factors, enzymes involved in neurotransmitter biosynthesis, neuropeptides, and other factors have been suggested to play important roles. Hawkins, R.D. *et al.*, *Annu. Rev. Neurosci.* 16:625-665 (1993); Grant, S.G. & Silva, A.J., *Trends in Neurosciences* 17:71-75 (1994); Alberni, C.M. *et al.*, *Cell* 76:1099-1114 (1994); Mello, C.V. & Clayton, D.F., *J. Neurobiol.* 26:145-161 (1995); Huston, J.P. & Hasenohrl, R.U., *Behav. Brain Res.* 66, 117-127 (1995); Zhuo, M. *et al.*, *Nature* 368:635-639 (1994). The instant invention has isolated a new *Drosophila* memory gene, *Volado* (*Vol*), that encodes a novel α-integrin, a type of cell surface receptor known to dynamically mediate cell adhesion and signal transduction. Hynes, R.O., *Cell* 69:11-25 (1992). "Volado" is

a Chilean colloquialism with no English counterpart, but is loosely translated as "forgetful" or "absent-minded." In Chile, it is often used in reference to professors and scientists.

Lesions in *Vol* have a dominant effect upon short-term memory following olfactory conditioning. Remarkably, conditional expression of *Vol* just before training rescues the memory deficit of *Vol* mutants. This rescue is reversible, supporting a dynamic role for integrins in neuronal and behavioral plasticity. These data indicate that integrin-mediated signaling or synaptic restructuring underlie the formation, stability, or retrieval of short-term memory.

There is a pressing need for the development of new cognitive enhancers. Our abilities to learn, and to forget, are human attributes often taken for granted because they operate in the background of our everyday tasks. Their importance surfaces when these abilities are compromised by head trauma, psychiatric or neurological disease, aging, alcoholism, or from other causes. As many as 5% of school-aged children experience grave difficulties with learning to read and spell. Memory disorders are common among the aged, affecting an estimated 12% of the population over age 65. The personal and financial costs of these disorders are staggering, with the cost of Alzheimer's disease alone at between 80-90 billion dollars each year. Therefore, it is incredibly important to discover effective treatments and cures for the numerous types of cognitive disease.

Despite this obvious need, there are few drugs on the market or in development that have a significant impact upon learning and memory cognitive processes. This is because the mechanisms underlying cognitive processes are complex and the many possible molecular targets have yet to be identified. This perhaps explains the diversity of diseases that affect cognitive processes. It also explains why the pharmaceuticals available are now directed toward enhancing the biological activity of only a handful of molecules. Given the likelihood that perhaps one

hundred different molecules are involved in cognitive processes, any of these, once identified, could potentially be modulated to enhance cognition with appropriate pharmaceuticals.

This discovery deals with the identification of a new molecule involved in cognitive processes. Using a novel methodology to identify genes and molecules involved in Drosophila cognitive processes, molecules of the *volado* family of proteins which are essential for normal cognitive processes formation were discovered. These proteins work by participation in signal transduction cascades and principally, in modulating the activity of tyrosine kinase.

This discovery now makes possible the use of expression systems of *volado* genes to identify pharmaceuticals that increase or decrease their activity.

SUMMARY OF THE INVENTION

An object of the present invention is a screening system for determining cognitive enhancers using the *Vol* protein in test cells.

An additional object of the present invention is a method for screening for cognitive enhancers using *Vol* or its corresponding mutants.

An additional object of the invention is using integrins or other analogous proteins as substitutes for use of the *Vol* proteins.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention, a method for detecting cognitive enhancers comprising the steps of inserting a gene sequence encoding for *Vol* protein into test cells in culture under conditions where said gene sequence expresses the *Vol* protein in said test cells; adding a test compound to the cell cultures or cell homogenates; and measuring the effect of the test compound on the activity of the *Vol* protein.

In specific embodiments of the present invention, the test cells are selected from a group of invertebrate cells and vertebrate cells. More specifically, they can be mammalian cells selected from the group consisting of human embryonic kidney cells, COS cells or CHO cells, or insect cells selected from Drosophila S2 or Spodoptera SF9 cells with baculovirus vectors.

The specific methods for measuring the activity on *Vol* protein include cell adhesion assays to ligands applied to solid surfaces such as plastic microtiter wells, or to ligands expressed on other cells, in which case the activity can be measured by cell aggregation. Additional specific embodiments use increases in tyrosine phosphorylation within cells expressing the integrin as a measure of integrin activity.

In another specific embodiment of the present invention, the cognitive enhancers are screened by using *volado* mutant Drosophila flies.

Other and further objects, features, and advantages will be apparent from the following description of the presently preferred embodiments of the invention, which are given for the purpose of disclosure, when taken in conjunction with the accompanying drawings.

Description of the Drawings

Figure 1 shows the *Vol* gene structure, transcripts and mutations. Figure 1A shows an EcoRI (R) restriction map of the locus with the position of the Vol^1 enhancer detector element indicated by a triangle. The direction of transcription of the *lacZ* reporter in the enhancer detector element is indicated by the arrow. Two transcription units, *Vol*-long (*Vol*-l) and *Vol*-short (*Vol*-s), were deduced by comparing cDNA sequences with genomic sequences. The first exon of each transcription unit is spliced to a common 2nd exon. Filled boxes represent the open reading frame. The 816 base pair deletion in Vol^2 is indicated by the line spanning the first exon of *Vol*-s. Figure 1B shows blots of adult head RNA showing the 4.6 and 4.4 kb transcripts of *Vol*-l and

Vol-s, respectively, in Canton-S (cs) and *ry* animals. Figure 1C shows reverse transcriptase (RT)-PCR analyses of total head RNA from rosy (*ry*), Vol^1 and Vol^2 adults. Each graded bar represents increasing amounts (from left to right) of a single RT reaction added to the subsequent PCR. Both *Vol*-l and *Vol*-s were present in *ry*; however, the expression of *Vol*-l was dramatically reduced in Vol^1 and expression of *Vol*-s was undetectable in Vol^2. The internal control using PKA primers allowed quantitative comparisons to be made between the various RT-PCR reactions. RNase (+) added prior to the RT reaction abolished all signals.

Figures 2A and 2B show *Vol* preferentially expressed in mushroom bodies. Figure 2A shows a frontal section of a Vol^1 adult head stained for β-galactosidase activity. Staining (blue signal) was observed within the mushroom body perikarya (mb). The β-galactosidase encoded by the enhancer detector element carried a nuclear targeting sequence which explains the nuclear localization of the histochemical stain. Figures 2B-2D show frontal sections of Canton-S adults after immuno-staining with an affinity-purified antiserum raised against the carboxy-terminus of *Vol*. Figure 2B shows expression (dark brown signal) observed in the cell bodies (mb) and calyces (c). Figure 2C shows the peduncle (p). Figure 2D shows the α, β, and γ lobes (α, β, γ).

Figure 3 shows memory deficits in *Vol* mutants. Figure 3A shows the decay curve of conditioned odor avoidance for two *Vol* mutants (Vol^1 and Vol^2) and the control strain (*ry*). N = 8 to 9 for all groups. The mean performance index ± SEM is shown for each genotype at several time points after training. The performance of Vol^1 and Vol^2 was significantly less than *ry* at all time points. Figure 3B shows the performance of homozygous and heterozygous *Vol* mutants at 3 and 15 minutes after training. N = 8 to 11 for all groups. There were no significant differences between the homozygous mutant strains and the corresponding heterozygous strains at either time point.

Figure 4 shows the lack of neuroanatomical defects in *Vol* mutants. *Ry* and *Vol*^{2} adult frontal sections are shown at the level of the mushroom body perikarya (mb) and calyces (c) after staining with hematoxylin and eosin (H&E) or with an antibody against the nuclear antigen D-mef2, and at the level of the mushroom body lobes (α, β and γ) after staining with anti-*fasII* or anti-*leonardo* antisera. No differences between the genotypes were observed in either mutant. Slight differences seen here were due to the plane of sectioning. The posterior to anterior arrangement of sections is from top to bottom.

Figure 5 shows rescue of the *Vol* memory defect by conditional expression of *Vol*-s. Figure 5A shows three minute memory without heat shock (NO HS) or 3 h after heat shock (HS 3h) in *ry*, *Vol*^{2}, VS-T2 and VS-T3. Heat shock was for 15 minutes at 37°C. N = 6 for all groups. Rescue of the mutant phenotype was exhibited by both VS-T2 and VS-T3; in addition, VS-T2 exhibited some constitutive rescue. Figure 5B shows RT-PCR analyses of *Vol*-s expression. RT-PCR for *ry*, VS-T2 and VS-T3 without (-) or 30 minutes after HS (+). Figure 5B upper panels show *ry* control: HS had no effect on expression of *Vol*-s or PKA in *ry* animals (compare duplicate lanes 1 and 2 with lane 7, all of which are from PCR reactions containing equivalent amounts of input cDNA). Quantitation using a BetaGen blot analyzer demonstrated that the signals for both *Vol*-s and PKA were linear with the mass of input cDNA (graded bar) amplified by PCR. Figure 5B lower panels show *Vol* transgenics: *Vol*-s RNA was nearly undetectable in both VS-T2 and VS-T3 in the absence of HS (-). Thirty minutes after HS (+) there was a marked induction of the transgene. Lanes 1, 2 and 7 in the upper panels and all lanes in the lower panels are from PCR reactions seeded with equivalent amounts of input cDNA. RNase treatment (+) prior to RT eliminates all signals. Data are representative of three independent experiments. Figure 5C shows *Vol* protein was induced after HS in VS-T3. Immunoblotting was performed on extracts from whole flies without (0h) or 3 and 24 hours after HS. Western blots containing

0.5 fly equivalents per lane were incubated with an affinity-purified antiserum generated against the carboxy-terminus of the *Vol* integrin. This antiserum recognizes both the full-length protein (~135 kDa) as well as the light chain (doublet at ~26 kDa). These data, confirmed by detection with an antiserum generated against the extracellular domain (not shown), are representative of 2 experiments. Figure 5D shows three minute memory without HS (NO HS) or 3 (HS 3h) and 24 h (HS 24h) after HS. N = 6 for all groups. VS-T3 showed a behavioral deficit without heat shock, normal performance with HS 3 h prior to training, but a deficit again when HS was given 24 h prior to training. Figure 5E shows RT-PCR analyses of *Vol*-s RNA expression without (0h) or 0.5 and 21.5 h after HS. Figure 5E upper panels show expression of *Vol*-s was not changed after HS in *ry* animals. Figure 5E lower panels show *Vol*-s RNA was dramatically elevated in VS-T3 0.5 h after HS, and returns to a low level at 21.5 h after HS. As in Figure 5B PKA expression was not changed by HS in either strain. All lanes are from PCR reactions seeded with equivalent amounts of input cDNA. RNase treatment prior to RT eliminates all signals. Data, from a single experiment performed in duplicate, are representative of two independent experiments.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned as well as those inherent therein. The *volado* and integrin proteins along with the DNA and protein sequences, methods, procedures, assays, molecules and specific compounds described herein are presently representative of the preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the claims.

Detailed Description of the Invention

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

The term "cognitive processes" as used herein refers to all aspects of intellectual ability, including the elements of problem solving, memory, levels of consciousness, orientation, attention and mental tracking, comprehension, judgment, calculations, reasoning, perception, planning, and constructional ability.

The term "cognitive enhancers" as used herein refers to any agent (for example a compound, composition or drug) that modifies the efficacy of one or more of the elements of the cognitive processes.

The term "mutant" as used herein refers to an alteration of the primary sequence of *volado* thus that it differs from the wild type or naturally occurring sequence. In the nucleic acid sequence, mutant can be any change in the sequence, for example changed base, deletion, or addition which results in an altered protein. In the amino acid sequence, the mutant is a peptide or protein whose sequence is altered from the native sequence.

The term "expression system" as used herein refers to a vector, plasmid or cell that contains all the information necessary to produce a protein from the *volado* gene sequence in *Drosophila* or its corresponding sequence from a mammalian species.

The term "transfection/transfected" as used herein describes the process of directly introducing the DNA into cells including vertebrate cells, invertebrate cells, bacteria and yeast. This includes introducing DNA by transfection of insect cells and mammalian cells.

As used herein, the term "transform/transformation" refers to the result of introducing DNA into a cell where the presence of the DNA genotypically and phenotypically alters a cell in a heritable manner.

The term "reporter" refers to the insertion of a nucleotide sequence downstream from a promoter such that when the promoter is activated the nucleotide sequence is produced in the cell. To be an effective reporter the nucleotide sequence must produce a peptide, protein or other change which can be monitored. For example, it could produce a protein which causes the cells to change color or can be linked to some type of enzyme or antibody reaction in order to detect the presence of the reporter. A skilled artisan readily recognizes that a variety of reporter genes are available for use in the present invention.

The term "*Volado*" as used herein refers to the gene in *Drosophila* which encodes the proteins identified in SEQ ID NOS. 1 and 2. A schematic of the gene and certain mutants is shown in Figure 1.

The terms "Vol^1" and "Vol^2" as used herein refer to the two *Volado* mutants. A schematic of these two mutants is shown in Figure 1.

One aspect of the present invention is the use of *Volado* proteins to screen for compounds, compositions or drugs to be used as cognitive enhancers. Any agent that increases the biochemical activity of *Volado* proteins in neurons could make cognitive processes more efficient, especially in cases of disease in with the activity of *Volado* proteins is compromised. Clones for the *Drosophila Volado* protein or its mammalian counterparts are used in expression systems for screening new agents that alter the biochemical function of *Volado* proteins.

One skilled in the art readily recognizes that a variety of expression systems can be used. These expression systems can be selected from either invertebrate cells, vertebrate cells, bacteria or yeast.

For example, the expression system can be comprised of mammalian cells. Examples of mammalian cells which are useful in the present invention include mammalian cells in culture, such as human embryonic kidney cells, COS cells, or CHO cells. Examples of invertebrate cells which can be useful in the present invention include insect cells in culture, such as *Drosophila* S2 cells or *Spodoptera* SF9 cells with baculovirus vectors.

Agents that alter the activity of *volado* or integrins are screened for in several different ways. After expression of a *volado* or integrin gene in cultured cells, agents are applied and their ability to modulate *volado* or integrin function is determined by: (1) cell adhesion assays since integrins mediate adhesive functions of cells. These include the binding of expressing cells to ligands deposited on plastic surfaces in microtitre wells or other solid surfaces, or cell aggregation assays in which integrin expressing cells adhere to cells expressing ligands for the integrins, (2) increases in tyrosine phosphorylation, since activation of integrins leads to increased levels of tyrosine kinase. *Drosophila* mutants that have lowered *volado* activity sufficient to cause lethality, poor viability, or learning/memory deficiency are used to screen for agents that alter the *volado* or integrin activity. Agents with this property rescue the viability defects and/or the defects in learning/memory *Drosophila* mutants that have a lowered *volado* activity sufficient to cause lethality, poor viability, or learning/memory deficiency are used to screen for agents that alter the *volado* or integrin activity. Agents with this property rescue the viability defects and/or the defects in learning/memory.

The predicted amino acid sequence of *Vol*-l and *Vol*-s are indicated as SEQ ID NO. 1 and SEQ ID NO. 2 respectively. The complete amino acid sequence for *Vol*-l is contained in SEQ ID NO. 1. The first 63 amino acids of *Vol*-s are listed as SEQ ID NO. 2.

Example 1
The *Vol* Locus Encodes a Novel α-integrin

Approximately 6000 enhancer detector lines were constructed and screened for preferential expression of the *lac*Z reporter in brain structures as is well known in the art. (*See e.g.*, Han, P.-L. *et al.*, *J. Neurobiol.* 31:88-102 (1996)). About 100 lines with preferential mushroom body expression were isolated, including insertions at the *dnc*, *rut*, *DCO* and *leonardo* (*leo*) genes. Line 1116 (Vol^1) from this screen also expressed *lac*Z in mushroom bodies; the enhancer detector element in this line was mapped to cytological position 51E.

The region flanking the enhancer detector element was isolated along with wild type genomic and cDNA clones for the locus. The locus is organized into two transcription units, *Vol*-long (*Vol*-l) and *Vol*-short (*Vol*-s), which encode RNAs of 4.6 and 4.4 kb, respectively (FIG. 1). The *Vol*-l RNA is expressed selectively in heads, whereas *Vol*-s is expressed in both head and body tissues. Mapping experiments showed that the Vol^1 enhancer detector element resides within the first intron of *Vol*-l and within the 5' flanking region of *Vol*-s (FIG. 1A). Imprecise excision of the element led to the isolation of Vol^2, an allele with an 816 nucleotide deletion of genomic sequence that removes the first exon of *Vol*-s (FIG. 1A). Reverse-transcriptase (RT)-PCR analyses of head RNA revealed that expression of the *Vol*-l transcript was greatly reduced in Vol^1, while the *Vol*-s transcript was unaffected (FIG. 1C). Conversely, the Vol^2 lesion eliminated the *Vol*-s transcript without discernible changes in *Vol*-l (FIG. 1C). Neither allele affected the expression of PKA, the internal control in these experiments (FIG. 1C). The effects of the alleles on expression of the two transcripts, as confirmed by RNA blotting experiments are consistent with the nature of the physical lesions at the gene (FIG. 1A). Thus, the Vol^1 and Vol^2 alleles disrupt *Vol*-l and *Vol*-s expression, respectively.

The cDNAs for *Vol*-l and *Vol*-s predict novel α-integrins of 1115 amino acids differing only in the first 63 amino acids (Fig. 1A, SEQ ID NO. 1, SEQ ID NO. 2). The *Vol* proteins contain many hallmarks of other α-integrins. The *Vol* proteins are 23-28% identical in amino acid sequence with known α-integrins and contain a single transmembrane domain near the carboxy-terminus. The proteins begin with 24 residues of a hydrophobic, putative signal peptide, have 11 potential glycosylation sites [NXT(S)] in the extracellular region, and have three repeats in the extracellular region that match the consensus DX(D/N)X(D/N)GXXD, which is well known in the art to be a domain found in proteins that bind divalent cations. (*See e.g.*,Kretsinger, R.H., *CRC Critical Reviews in Biochemistry* 8:119-74 (1980)). Moreover, the *Vol* sequence has a cleavage recognition site (RKRR) in the extracellular domain, a site required for signal transduction by some α-integrins. After cleavage at these sites, the amino-terminal and carboxy-terminal integrin fragments are held together by disulfide bonds. Furthermore, the cytoplasmic domain of *Vol* contains the consensus sequence, KXFF[K/R]R, which is known in the art to bind calreticulin (*See e.g.*, Dedhar, S., *Trends in Biochem. Sci.* 19:269-307 (1994)) and regulate integrin affinity for ligand.

Example 2
Expression of *Vol* in Mushroom Bodies

The Vol^1 mutant preferentially expressed the *lacZ* reporter in the nuclei of mushroom body neurons (FIG. 2A). To determine if the enhancer detector reflected authentic *Vol* protein expression, immunohistochemical analyses with an antiserum made against the carboxy-terminus of the protein were performed. The *Vol* antigen was found to be concentrated in the mushroom body perikarya and calyces (FIG. 2B), peduncles (FIG. 2C), and α, β, and γ lobes (FIG. 2D). The calyces, peduncles, and lobes contain the mushroom body dendrites, axons, and axon terminals,

respectively. The distribution of the antigen was not noticeably altered in either the *Vol*^{1} or *Vol*^{2} mutants (data not shown), suggesting that both *Vol*-l and *Vol*-s isoforms are globally co-expressed in the mushroom bodies. Enriched expression was also observed in the ellipsoid body (not shown), a region of the central complex thought to be involved in the coordination of motor behaviors. The distribution of *Vol* in the mushroom body calyces and lobes - regions in which mushroom body neurons form synapses with other neurons suggests that the *Vol* integrins could regulate synapse function.

Example 3
Mutations in *Vol* Produce a Memory Deficit

The expression pattern of *Vol*, coupled with preliminary behavioral experiments, suggested that this gene is important for olfactory memory. To test this hypothesis, Vol mutants were assayed for aversive olfactory classical conditioning. Populations of animals were administered electric shock (unconditioned stimulus, US) in the presence of one odor, the conditioned stimulus (CS+), and were subsequently presented a second odor (CS-) without shock. To evaluate discriminative avoidance behavior, the trained animals were allowed to distribute between converging CS+ and CS- odors carried in air currents within a T-maze.

Animals homozygous for the *Vol*^{1} insertion or the *Vol*^{2} deletion performed poorly relative to *ry* at all time points after training (FIG. 3A; genotype, P = .0001; retention interval, P = .0001; genotype x retention interval, NS). The effects of these mutations on memory were indistinguishable, suggesting that the two integrin isoforms are functionally redundant. It had been shown previously that neither the enhancer detector itself, nor the expression of *lacZ* in mushroom bodies per se, have any significant effect upon performance. The performance deficits in *Vol* mutants were present at the earliest testable time point after training (3 minutes), indicating

that the formation, stability, or retrieval of short-term memory is dependent upon integrin function.

To further examine the effects of the *Vol* alleles on early memory and to investigate their recessive or dominant nature, the performance of animals heterozygous or homozygous for the two lesions of the gene was trained and tested. The Vol^1 and Vol^2 animals exhibited memory deficits at both 3 and 15 minutes after training (FIG. 3B; 3 minutes, P = .0001; 15 minutes, P = .0001), confirming the results in Figure 3A. The performance index (PI) of the Vol^1/+ and Vol^2/+ heterozygous animals was similarly reduced relative to *ry*, but was not significantly different from the corresponding homozygous mutants (FIG. 3B; Vol^1 vs. *ry* at 3 minutes, P = .0001; at 15 minutes, P = .0001; Vol^2 vs. *ry* at 3 minutes, P = .00015; at 15 minutes, P = .0001; Vol^1 vs. Vol^1/+ at 3 minutes, NS; at 15 minutes, NS; Vol^2 vs. Vol^2/+ at 3 minutes, NS; at 15 minutes, NS). Trans-heterozygous animals, Vol^1/Vol^2, also exhibited a PI equivalent to Vol^1/+ or Vol^2/+. Thus, as with *dnc*, *rut*, *turnip*, *radish*, and *cabbage*, mutations in *Vol* have a dominant effect on memory. The dominant effect is particularly noteworthy for *Vol* alleles, since three out of the four transcription units were preserved in animals heterozygous for *Vol*-l or *Vol*-s lesions. These data support the existence of a threshold requirement for *Vol* expression in the processes underlying memory, making them acutely sensitive to decreased expression of this gene.

Example 4
Evaluation of *Vol* Sensorimotor Processes

To eliminate the possibility that the poor performance of *Vol* mutants was due to defects in sensorimotor processes, their ability to sense and avoid electrical shock pulses and the odors used for conditioning was tested. The avoidance behavior of *Vol* mutants and control animals to electrified grids and odors used for conditioning at multiple strengths of these stimuli was

indistinguishable. For example, the avoidance indices to 0.8 ml octanol were 63±4, 68±4, and 65±5 for *ry*, *Vol*^{1} and *Vol*^{2}, respectively. The morphology of the brain was explored with a particular emphasis on mushroom bodies to determine whether the poor performance was attributable to defects in brain structure. Serial paraffin sections of control and mutant brains failed to reveal any discernible differences in morphology when stained with Hematoxylin and Eosin (H&E); an antibody against the nuclear antigen D-mef2, which reveals a subset of mushroom body cell nuclei; an antibody against the *leo* gene product, which delineates the mushroom body calyces, cell bodies, peduncles, and lobes, or an antibody against fasII, which reveals a subset of the mushroom body lobes (FIG. 4). Therefore, neither sensorimotor or gross neuroanatomical defects can account for the memory deficit of *Vol* mutants.

Example 5
Conditional Rescue of the *Vol* Memory Deficit

Direct evidence for a role of the integrin in physiological processes underlying memory is obtained through the conditional expression of a *Vol* transgene. Four transgenic lines were generated that harbored the *Vol*-s cDNA under the control of the hsp70 promoter in the *Vol*^{2} background (*Vol*-s mutant). Animals were heat-shocked for 15 minutes at 37°C, rested for 3 hours to allow for recovery and expression of the transgene, and subsequently trained and tested for 3 minute memory. Two of the transgenic lines failed to show any evidence of heat-dependent rescue in pilot experiments, presumably due to genomic position effects, and were not analyzed further. Two other lines, VS-T2 and VS-T3, were analyzed extensively for olfactory memory.

Normal olfactory memory of *ry* control animals and the residual memory in Vol^2 mutants was unaffected by heat shock (FIG. 5A; for *ry*, NO HS vs. HS 3h, NS; for Vol^2, NO HS vs. HS 3h, NS). In the absence of heat shock, VS-T3 transgenic animals exhibited mutant levels of

performance, but VS-T2 transgenic animals showed partial rescue of memory, possibly due to elevated basal expression of the transgene in mushroom bodies (FIG. 5A; NO HS, VS-T3 vs. *ry*, P = .0001; VS-T3 vs. Vol^2, NS; VS-T2 vs. *ry*, P = .0008; VS-T2 vs. Vol^2, P = .0003). However, the 3 minute memory of VS-T2 and VS-T3 animals when tested 3 hours after heat shock, was significantly improved over that after no heat shock and was indistinguishable from the *ry* control (FIG. 5A; HS vs. NO HS, VS-T2, P = .0045; VS-T3, P = .0005; with HS, *ry* vs. VS-T2, NS; *ry* vs. VS-T3, NS). Therefore, conditional expression of *Vol*-s just before behavioral training was sufficient to fully rescue the mutant phenotype. This rescue cannot be attributed to altered sensorimotor abilities, since avoidance behavior to electric shock and odors by the control and transgenic animals was indistinguishable, with or without heat shock. These data provide compelling evidence that the defective α-integrin expression in *Vol* mutants is responsible for the memory deficits, and that the *Vol* integrin participates in the physiological processes underlying memory.

To determine whether the behavioral rescue was paralleled by the induction of the *Vol* transgene, *Vol* RNA and protein levels were assayed before and after heat shock. As assayed by RT-PCR, heat shock had no effect on the quantity of *Vol*-s RNA in *ry* control animals (FIG. 5B), but produced a ~100-fold and ~1000-fold increase in the level of *Vol*-s RNA in the VS-T2 and VS-T3 transgenic lines, respectively (FIG. 5B). The level of PKA RNA served as an internal control and was unaffected by *Vol* mutation (FIG. 1B), *Vol* transgene expression, or heat shock (Fig. 5B). Western blotting was used to measure *Vol* protein using an affinity-purified antiserum raised against the carboxy-terminus of *Vol* that recognized the intact *Vol* protein (Mr ≈ 125 kDa), as well as the carboxy terminal cleavage fragment produced by proteolysis (Mr ≈ 21 kDa). This antiserum identified a band (sometimes a doublet) of 26 kDa in *ry* that was not found in Vol^2 mutants or in non-heat shocked transgenics animals (FIG. 5C). This band represents the

carboxy-terminal cleavage fragment. The full-length protein was not detected in *ry* extracts, presumably due to reduction of the disulfide bond that links the heavy and light chains. In contrast to the *ry* control, a large increase in the expression of both the *Vol* full-length protein and light chain was found in VS-T3 extracts obtained three hours after heat shock (FIG. 5C). Detection of the intact molecule suggests that the protease is limiting after over-expression of *Vol*. Induction of *Vol* protein was also observed in VS-T2. Thus, there was a marked elevation of the *Vol* α-integrin in the VS-T2 and VS-T3 transgenics 3 hours after heat shock. These RNA and protein analyses demonstrated that *Vol* was conditionally expressed at the time of behavioral assay, confirming that replacement of the *Vol* integrin in adulthood rescued the memory deficit.

Despite the arguments presented above for a physiological role for *Vol*, it seemed plausible that the α-integrin might be required for a final step in synapse formation that occurs normally during development, and that the induced expression of the integrin during adulthood simply allows completion of this terminal step. In other words, the presence of the integrin might be essential for synapse formation but not for synapse stability. If so, the induction of *Vol* expression might cause a long-lasting or permanent rescue of memory. If, on the other hand, *Vol* participates in a non-developmental, acute aspect of neuronal function, the rescue of memory produced by induction of the *Vol* transgene would be expected to be transient and reversible, persisting only as long as adequate levels of the *Vol* integrin are present.

To distinguish between these possibilities, whether induction of *Vol* produced a permanent or a reversible restoration of memory was explored. As before (FIG. 5A), heat shock treatment 3 hours prior to training and testing dramatically improved the performance of VS-T3 animals (FIG. 5D; for VS-T3, NO HS vs. HS 3h, $P = .0001$). This rescue was completely reversible. The memory in heat-shocked VS-T3 transgenic animals returned to mutant levels when the animals

were trained and tested 24 hours after heat shock (Fig. 5D; for VS-T3, no HS vs. HS 24h, NS; HS 3h vs. HS 24h, P ..0001; for HS 24h, *ry* vs. VS-T3, P = .0001; Vol^2 vs. VS-T3, NS).

Vol RNA and protein expression in the transgenic animals, which reflect abundance in all cells, were markedly elevated at early time points after heat shock (0.5 and 3 hours, respectively), and decreased to low levels at late time points (21.5 and 24 hours, respectively) (FIGS. 5C, 5E). Thus, the induction and ensuing decline of *Vol* expression correlated well with the behavioral rescue and subsequent return to a state of memory impairment. The temporal parallels in RNA level, protein expression, and memory argue strongly that *Vol* mediates a physiological process that is critical to memory formation, stability, or retrieval.

Collectively, these results support three important points. First, reduced expression of the *Vol* integrin produces an impairment in memory without altering sensorimotor abilities or neuroanatomy. Second, this phenotype is rescued by the expression of the integrin just before training in the adult animal, demonstrating an adult role for this adhesion molecule. Third, the reversibility of the memory rescue indicates that the *Vol* integrin mediates a dynamic process underlying memory.

Example 6
Integrins, Synaptic Plasticity, Mushroom Bodies and Memory

The results of the identification, isolation, and characterization of *Vol* properties of *Vol* similar to those seen in studies of four other learning genes with similar expression patterns: *dnc*, *rut*, *DCO* and *leo*. (For examples of other learning genes *see e.g.*, Davis, R.L., *Neuron* 11:1-14 (1993); Skoulakis, E.M.C. & Davis, R.L., *Neuron* 17:931-44 (1996). Nighorn, A. *et al.*, *Neuron* 6:455-467 (1991); Han, P.-L. *et al.*, *Neuron* 9:619-627 (1992); Skoulakis, E. *et al.*, *Neuron* 11:197-208 (1993)). The discovery of another memory mutant in which the underlying gene is

expressed preferentially in mushroom bodies reinforces the conclusion that these cells play a crucial role in olfactory learning and memory. The mushroom bodies may serve as centers for the reception and integration of many different forms of sensory information, including information about odors and electric shock presented during olfactory classical conditioning. The converging sensory information is thought to alter the physiology of mushroom body cells to encode memory, employing the cAMP signalling system as well as other types of molecules. The results with *Vol* demonstrate that integrins are included in the family of molecules required for memory formation.

Integrins have diverse biological roles in apoptosis, cell cycle regulation, cell migration, blood clotting and leukocyte function. They function as αβ heterodimers, mediating adhesive interactions of cells with the extracellular matrix or with counter-receptors displayed by other cells. Most interestingly, they dynamically transduce information across cell membranes bi-directionally. Ligand binding to integrins induces a variety of signalling events within cells, and agonist activation of classical signal transduction pathways can alter the affinity of integrins for their ligands within a time-frame of a few minutes.

The dynamic adhesion role for integrins offers a hypothesis for how the *Vol* integrin, and integrins in general, underlie alterations in synaptic plasticity and behavior. It is envisioned that release of a modulatory neurotransmitter upon a mushroom body neuron might mobilize the intracellular events leading to an altered binding of integrins displayed at another synapse made by that cell. For example, protein kinase C or ras activation is known to activate integrin binding. This could produce a rapid (within minutes) alteration in the structure and efficacy of that synapse. The modulation of integrin affinity for ligands might also underlie the construction or pruning of existing synapses, or the activation of silent synapses during learning or memory encoding. Thus, the formation of short-term memory may employ synaptic rearrangements like long-term memory, but through an integrin-dependent, and protein synthesis-independent

mechanism. Alternatively, it is possible that integrins modulate neuronal function through ligand binding followed by activation of intracellular signalling events. For example, integrins are known to stimulate a number of signal transduction pathways in many types of cells, including Ca^{2+} mobilization, tyrosine kinase activation, and induction of protein kinase C. Integrin-dependent stimulation of these pathways in the relevant neurons may be fundamental to learning and memory.

The results demonstrating a role for integrins in behavioral plasticity mesh well with studies showing integrin-dependent modulation of synaptic plasticity. Notably, peptide inhibitors of integrin binding have no effect upon the formation of long-term potentiation, but block the maintenance of this form of synaptic plasticity. In addition, the enhancement of neurotransmitter release from motor nerve terminals due to muscle stretch is blocked by the peptide inhibitors. Psychological studies coupled with these behavioral studies, support a model in which integrins mediate dynamic processes at synapses underlying memory formation or stability.

EXAMPLE 7
Cloning, Mutagenesis and Transgenic Animals

Genomic sequences flanking the *Vol*^{1} insertion were isolated by plasmid rescue. Wild-type genomic clones were isolated from a Canton-S library made in lambda DASHII; cDNA clones were isolated from libraries prepared from *Drosophila* head RNA. The 4.6 kb *Vol*-l RNA sequence is represented by a cDNA of ~4600 residues. The 4.4 kb *Vol*-s RNA is represented by a 3366 bp cDNA.

The *Vol*^{2} excision was isolated after dysgenesis. Flies carrying the *Vol*^{1} enhancer detector element were crossed to Xcs; CyO/2cs; *ry* Sb P[ry+,D2-3,99B]/TM6,Tb. ("cs" denotes chromosomes derived from a wildtype Canton-S stock.) Dysgenic progeny carrying CyO were

crossed to Xcs; CyO/leo^{1375}; ry^{506}-iso animals. CyO; ry^{506}-iso progeny were selected for stocks. ry^{506}-iso is an isogenic ry^{506} chromosome. Excision derivatives were characterized by Southern blotting, extensive PCR analyses, and sequencing of PCR products that cross deletion break points.

Due to the nonspecific behavioral effects of mini-white vectors, a new P-factor vector (pCy-20-dbhsp) for driving genes behind the hsp70 promoter was constructed with *ry*+ as the selectable marker. This vector, containing a MluI-KpnI fragment of the *Vol*-s cDNA was injected into *Vol*^{2} embryos. Chromosomal localization of the transgenes and the generation of homozygotes for the transgenes were performed by standard crosses. The presence of the *Vol*^{2} allele in the transgenic animals was confirmed by PCR analyses of genomic DNA. The *Vol* transgene resides on the X and 2nd chromosome, respectively, in VS-T2 and VS-T3.

Flies were collected in clean food vials, transferred to pre-warmed food vials, and immersed in a 37°C water bath for 15 minutes. Following heat-shock, flies were transferred to room-temperature food vials and stored until testing.

EXAMPLE 8
RNA Blots and RT-PCR Analyses

For RNA blots, polyA+ RNA was isolated after tissue homogenization in guanidinium-isothiocyanate, banding in CsCl gradients, and by batch adsorption to oligo-(dT) cellulose. Ten µg polyA+ RNA was fractionated per lane by formaldehyde-agarose gel electrophoresis. For RT-PCR experiments, total RNA from heads or whole flies was extracted using Trizol (Gibco-BRL) according to the manufacturer's instructions. Each RT reaction contained 3 µg total RNA, 500 ng oligo-(dT), and 200 U SuperScript II (Gibco-BRL) in a total volume of 20 µl. The reactions were incubated at 42°C for 50 minutes and digested with 10 U

*Sau*3AI and 10 U *Aci*I at 37°C for 3 hours. RNase A treatments (10 μg) prior to RT reactions were for 1 hour at 37°C. Aliquots of 0.2-5.0% of the RT reactions were amplified using PCR for 20 cycles. For amplification of *Vol* first-strand cDNAs, an antisense primer that anneals to the common 2nd exon of *Vol* (857 nucleotides 3' of translation start site) was used in combination with sense primers specific for the first exon of either *Vol*-l or *Vol*-s (84 and 118 nucleotides 5' to translation start site, respectively). For amplification of PKA, primers that anneal to the 2nd exon of *DCO* were used. PCR products (942, 975 and 356 bp for *Vol*-l, *Vol*-s and PKA, respectively) were electrophoresed in agarose gels, blotted, and hybridized to 32P-labelled probes.

EXAMPLE 9
Histology, Generation of Antisera, and Immunoblotting

β-galactosidase staining and H&E staining was performed as is known in the art. (*See e.g.*, Han, P.-L. *et al.*, *J. Neurobiol.* 31:88-102 (1996); Skoulakis, E.M.C. & Davis, R.L., *Neuron* 17:931-44 (1996); Han, P.-L. *et al.*, *Neuron* 9:619-627 (1992); Skoulakis, E.M. *et al.*, *Neuron* 11:197-208 (1993); Han, K.-A. *et al.*, *Neuron* 16:1127-35 (1996)).

For generation of antisera, rabbits were injected with a purified GST-*Vol* fusion protein containing either *Vol* amino acid sequence 1087-1115 (carboxy-terminus) or 358-496 (extracellular domain). For immunohistochemistry using anti-*Vol* antisera and the anti-fasII monoclonal antibody 1D4, adult heads were fixed in 4% paraformaldehyde at 4°C for 2 hours and incubated in 25% sucrose in Ringer's solution at 4°C overnight. Ten mm serial cryosections were incubated with affinity-purified anti-Vol or anti-fasII antibody at 4°C overnight. For anti-D-mef2 and anti-leonardo staining, adult heads were fixed in Carnoy's for 4 hours, embedded in paraffin, sectioned and incubated with the appropriate antiserum overnight at 23°C. In all cases, the antigen/antibody complexes were visualized using the Elite Vectastain ABC kit (Vector

Laboratories). For immunoblotting, protein extracts were prepared by homogenizing whole flies in 2X Laemli's sample buffer containing 1% β-mercaptoethanol at 75°C for 30 minutes. Fly extracts (0.5 fly equivalents per lane) were electrophoresed on SDS-polyacrylamide gels and blotted onto PVDF membranes (Millipore). Blots were incubated with affinity-purified anti-*Vol* sera overnight at 4°C, HRP-conjugated goat-anti-rabbit IgG (Jackson Laboratories) for 1 hour at 23°C, and visualized with SuperSignal Chemiluminescent substrate (Pierce).

EXAMPLE 10
Behavioral Analyses

The differential olfactory conditioning paradigm pairing the presentation of one odor with aversive shock and a second odor with the absence of shock, was used to assess learning and memory performance. Training and testing were performed blind to strain under dim red light at 23-25°C and 63-68% relative humidity using procedures well known in the art. (*See e.g.*, Skoulakis, E.M.C. & Davis, R.L., *Neuron* 17:931-944 (1996)). In each group, a performance index (PI) was calculated as the fraction of flies that avoided the CS+ minus the fraction of flies that avoided the CS-, and multiplied by 100. In practice, PI scores ranged from 0 (naive behavior) to 100 (perfect performance). Because the minimum possible time between training and testing is 3 minutes (due to handling and recovery of flies after transfer), 3 minute memory reflects the earliest testable time point. To test longer-term memory, the flies were returned as a group to their collection vials for the appropriate retention interval and then tested as above. Odor avoidance was calculated as the fraction of flies that avoided the odor in one arm minus the fraction of flies that avoided fresh air (and multiplied by 100) provided in the control arm. Electroshock avoidance was calculated similarly.

EXAMPLE 11
Statistics

Statistical analyses were performed with Statview 2.0 (Abacus Concepts, Berkeley, CA). Overall ANOVAs were followed by planned comparisons contrasting the relevant groups. Error rate due to multiple comparisons was controlled by dividing the alpha level by the number of comparisons being performed on a given set of data.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: DAVIS, Ronald L.
ZHU, Xin-Ran
GROTEWIEL, Michael S.
BECK, Christine D. O.
WU, Kwok-Hang

(ii) TITLE OF INVENTION: Method of Screening for Neuropharmaceuticals Using the Drosophila Gene Volado and Its Mutants

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fulbright & Jaworski L.L.P.
(B) STREET: 1301 McKinney, Suite 5100
(C) CITY: Houston
(D) STATE: Texas
(E) COUNTRY: U.S.A.
(F) ZIP: 77010-3095

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Paul, Thomas D.
(B) REGISTRATION NUMBER: 32,714
(C) REFERENCE/DOCKET NUMBER: P-01467US0

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 713/651-5325
(B) TELEFAX: 713/651-5246

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1115 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Ala Leu Ile Ser His Ile Glu Ala Phe Asn Phe Met Pro Arg Pro Ser
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Arg Val Ile Asn Ser Pro Lys His Leu Lys Phe His Ile Asn Gln Thr
        35                  40                  45

Arg Ser Ser Tyr Phe Gly Tyr Thr Leu Val Ile Arg Gln Thr Ser Ile
    50                  55                  60

Ile Val Gly Ala Pro Arg Ala Gln Ser Thr Leu Glu Ser Gln Arg Thr
65                  70                  75                  80

Ile Asn Glu Thr Gly Ala Ile Tyr Arg Cys Ser Leu Thr Asn Gly Val
                85                  90                  95

Cys Ser Pro Tyr Val Leu Asp Ser Arg Gly Asn Val Asp Ala Pro Tyr
            100                 105                 110

Ser Glu Tyr Thr Phe Asp Ser Glu Arg Lys Asp Phe Gln Trp Leu Gly
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Gly Ser Met Asp Gly Gly Thr Lys Asp Thr Asp Lys Leu Leu Val Cys
    130                 135                 140

Ala Pro Arg Phe Tyr Ala Pro Ser Ser Arg Asp Asn His Leu His Gly
145                 150                 155                 160

Val Cys Tyr Trp Val Asn Asn Thr Val Ala Ser Thr Pro Gln His Val
                165                 170                 175

Thr Arg Ile Ser Pro Leu Arg Leu Lys Ser Glu Gln Val Lys Glu Glu
            180                 185                 190

Asp Asn Gly Asn Lys Ala Ser Phe Phe Tyr Ile Met Gly Glu Leu Gly
        195                 200                 205

Leu Ser Ala His Val Ala Asp Asp Asn Thr Lys Phe Leu Ile Gly Ala
    210                 215                 220

Pro Gly Ile Asn Thr Trp Arg Gly Ser Val Ile Leu Tyr Arg Gln Val
225                 230                 235                 240

Asp Pro Val Asp Asn Pro Thr Ala Ser Arg Arg Asp Thr Ser Lys Ala
                245                 250                 255

Leu Arg Arg Thr Tyr Arg Asp Val Asp Ser Asn Asp Tyr Thr Pro Glu
            260                 265                 270
```

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His Tyr Ala Pro Glu Ile Pro Thr Pro Gly Leu Trp Gly Gln Glu Glu
        275                 280                 285

Asp Ser Tyr Phe Gly Tyr Ala Val Ser Ser Gly Phe Phe Asp Ser Ser
    290                 295                 300

Asn Pro Thr Lys Leu Leu Tyr Val Ala Thr Ala Pro Gln Ala Asn Lys
305                 310                 315                 320

Gln Ser Gly Glu Ala Tyr Ile Phe Asp Val Arg Gly Lys Ser Ile His
                325                 330                 335

Lys Tyr His Val Phe Arg Gly Glu Gln Phe Gly Glu Tyr Phe Gly Tyr
            340                 345                 350

Ser Val Leu Ala Glu Asp Leu Asn Gly Asp Gly Lys Thr Asp Val Ile
        355                 360                 365

Val Ser Ala Pro Gln His Ala Leu Glu Asp Ser His Asp Asn Gly Ala
    370                 375                 380

Ile Tyr Val Phe Ile Asn Lys Gly Phe Phe Asn Phe Glu Arg Gln Ile
385                 390                 395                 400

Leu Arg Ser Pro Val Glu Thr Met Ala Arg Phe Gly Thr Ala Leu Ser
                405                 410                 415

Arg Leu Gly Asp Ile Asn His Asp Gly Tyr Asn Asp Val Ala Val Gly
            420                 425                 430

Ala Pro Phe Ala Gly Asn Gly Thr Val Phe Ile Tyr Leu Gly Ser Glu
        435                 440                 445

Asn Gly Leu Arg Asp Gln Pro Ser Gln Arg Leu Asp Ala Pro Ser Gln
    450                 455                 460

Gln Pro Ser Lys Tyr Gly Ser His Met Phe Gly His Gly Leu Ser Arg
465                 470                 475                 480

Gly Ser Asp Ile Asp Gly Asn Gly Phe Asn Asp Phe Ala Ile Gly Ala
                485                 490                 495

Pro Asn Ala Glu Ala Val Tyr Leu Tyr Arg Ala Tyr Pro Val Val Lys
            500                 505                 510

Val His Ala Thr Val Lys Ser Glu Ser Arg Glu Ile Lys Pro Glu Gln
        515                 520                 525

Glu Lys Val Lys Ile Thr Ala Cys Tyr Arg Leu Ser Thr Thr Ser Thr
    530                 535                 540

Asp Lys Leu Val Gln Glu Gln Glu Leu Ala Ile Arg Ile Ala Met Asp
545                 550                 555                 560

Lys Gln Leu Lys Arg Val Lys Phe Thr Gln Thr Gln Thr Asn Glu Ile
                565                 570                 575
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```
Ser Phe Lys Val Asn Ala Asn Phe Gly Glu Gln Cys Arg Asp Phe Glu
            580                 585                 590

Thr Gln Val Arg Tyr Ser Glu Lys Asp Ile Phe Thr Pro Ile Asp Leu
        595                 600                 605

Glu Met His Tyr Glu Leu Thr Lys Lys Val Pro Asp Ser Glu Glu Phe
    610                 615                 620

Cys Glu Thr Cys Ala Val Val Asp Pro Thr Glu Pro Lys Val Ser Thr
625                 630                 635                 640

Gln Asn Ile Ile Phe Ser Thr Gly Cys Ala Thr Asp Val Cys Thr Ala
                645                 650                 655

Asp Leu Gln Leu Arg Ser Lys Asn Val Ser Pro Thr Tyr Ile Leu Gly
            660                 665                 670

Ser Ala Asp Thr Leu Arg Leu Asn Tyr Glu Ile Thr Asn Ile Gly Glu
        675                 680                 685

Thr Ala Tyr Leu Pro Gln Phe Asn Val Thr Ser Thr Ser Arg Leu Ala
    690                 695                 700

Phe Ala Gln Val Pro Gly Asn Cys Lys Val Val Asp Ala Val Met Val
705                 710                 715                 720

Cys Asp Leu Asn Arg Gly Arg Pro Leu Ala Lys Gly Asp Thr Asp Ser
                725                 730                 735

Val Thr Ile Ser Phe Asp Val Ser Gln Leu Ser Gly Gln Ser Leu Ile
            740                 745                 750

Ser His Ala Glu Val Phe Ser Thr Gly Tyr Glu Gln Asn Pro Thr Asp
        755                 760                 765

Asn Arg Gln Thr Asn Val Ile Gly Leu Lys Glu Phe Thr Glu Ile Asp
    770                 775                 780

Ala Ser Gly Gly Gln Thr Asn Arg Gln Ile Asp Leu Glu His Tyr Ser
785                 790                 795                 800

Asn Ser Ala Glu Ile Val Asn Asn Tyr Glu Ile Lys Ser Asn Gly Pro
                805                 810                 815

Ser Val Ile Glu Gln Leu Thr Val Ser Phe Tyr Ile Pro Ile Ala Tyr
            820                 825                 830

Lys Val Ala Gly Ser Thr Ala Ile Ile Pro Ile Ile Asn Val Thr Ser
        835                 840                 845

Leu Lys Met Gln Ala Ser Tyr Asp Ser Gln Leu Leu Ser Ile Asp Leu
    850                 855                 860

Tyr Asp Gln Asn Asn Thr Met Leu Val Val Asp Pro Val Glu Val Thr
865                 870                 875                 880
```

```
Thr Thr Leu Ser Gly Gly Leu Glu Arg Thr Val Ile Thr Gln Asn Arg
                885                 890                 895

Gln Ser Tyr Asp Ile His Thr Ser Gly His Val His Gln Thr Met Glu
            900                 905                 910

Val Leu Asp Thr Ser Met Val Ala Thr Ala Ser Met Ser Arg Lys Arg
        915                 920                 925

Arg Asp Leu Lys Ala Leu Thr Ala Asn Arg Glu Gln Tyr Ala Arg Ile
    930                 935                 940

Ser Asn Val Lys Ala His Asp Leu Leu Ser Asp Asp Phe Lys Gly Lys
945                 950                 955                 960

Leu Pro Val Asn Arg Thr Ile Val Phe Asn Cys Arg Asp Pro Glu Met
                965                 970                 975

Thr Ile Cys Val Arg Ala Glu Met Arg Val His Phe Arg Pro Glu Lys
            980                 985                 990

Ser Ile Asn Leu Asn Met Arg Tyr Ser Val Asp Leu Asn Glu Val Asn
        995                 1000                1005

Ala Ile Leu Val Asp Pro Trp Glu Tyr Phe Val Ile Leu Thr Asp Leu
    1010                1015                1020

Lys Leu Gln Lys Lys Gly Asp Pro Thr Ser Thr Ser Phe Ser Ile Asn
1025                1030                1035                1040

Arg Arg Ile Glu Pro Asn Ile Ile Ser Lys His Gln Glu Thr Gly Leu
                1045                1050                1055

Pro Ile Trp Ile Ile Ile Val Ser Val Ile Gly Gly Leu Leu Leu Leu
            1060                1065                1070

Ser Ala Ile Ser Tyr Leu Leu Tyr Lys Phe Gly Phe Phe Asn Arg Thr
        1075                1080                1085

Lys Lys Asp Glu Leu Asp Arg Leu Val Gln Gln Asn Pro Val Glu Pro
    1090                1095                1100

Glu Ala Glu Asn Leu Asn Ser Gly Gly Asn Asn
1105                1110                1115
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Val Gly Gln Asp Arg Asp Phe Trp Ala Leu Leu Val Leu Gly Leu
1               5                  10                  15

Trp Cys Leu Ser Ser His Cys Asn Ala Phe Asn Leu Ser Pro Leu Pro
            20                  25                  30

Asn Arg Gln Ile Leu Asp Pro Gln Phe Ala Thr Asn Leu Pro Lys Val
        35                  40                  45

Arg Ala Ser Tyr Phe Gly Phe Thr Met Ser Leu Arg Pro Asn Gly
    50                  55                  60
```

We claim:

1. A method for detecting a test compound for cognitive enhancer activity, comprising the steps of:

inserting a gene sequence encoding for a protein involved in cognitive processes into test cells in culture under conditions where said gene sequence expresses the protein involved in cognitive processes in said test cell;

adding the test compound to the cell culture or cell homogenates; and

measuring the effect of the test compound on the activity of the protein involved in cognitive processes.

2. The method of claim 1, wherein the protein is a *Volado* protein.

3. The method of claim 1, wherein the protein is an integrin protein.

4. The method of claim 1, 2, or 3 wherein the test cells are selected from the group consisting of invertebrate cells and vertebrate cells..

5. The method of claim 1, 2, or 3 wherein the test cells are mammalian cells.

6. The method of claim 5, wherein the mammalian cells are selected from the group consisting of human embryonic kidney cells, COS cells and CHO cells.

7. The method of claim 1, 2, or 3 wherein the cells are insect cells.

8. The method of claim 7, wherein the cells are selected from the group consisting of *Drosophila* S2 cells and *Spodoptera* SF9 cells with baculovirus vectors.

9. The method of claim 1, 2, or 3 wherein the measuring step includes determining the degree of cell adhesion to ligands applied to solid surfaces.

10. The method of claim 9 wherein the solid surface is a plastic microtiter well.

11. The method of claim 1, 2, or 3 wherein the measuring step includes determining the degree of cell adhesion to ligands expressed on other cells.

12. The method of claim 1, 2, or 3 wherein the measuring step includes biochemical assays of activity of tyrosine kinase.

13. The method of claim 2, wherein the gene expressing said *volado* protein is from a vertebrate or an invertebrate.

14. The method of claim 3, wherein the gene expressing said integrin protein is from a vertebrate or an invertebrate.

5. A method of detecting a test compound for cognitive enhancer activity, comprising the steps of:

feeding Drosophila flies the test compound; and

testing the treated Drosophila flies for effects on their olfactory learning and memory.

16. The method of claim 15, wherein the Drosophila flies are wild type flies or *volado* mutants.

17. The method of claim 13, wherein the gene is from Drosophila.

18. The method of claim 13 or 14, wherein the gene is from a mammal.

19. The method of claim 16, wherein said *Volado* mutant is selected from the group consisting of vol^1 and vol^2.

20. An Antibody against a *Volado* protein.

21. An Antibody against an integrin protein.

22. A method of screening for mutant Drosophila flies involved in cognitive processes comprising the steps of:

making a plurality of Drosophila fly line, each line containing a transposable element linked to a reporter gene;

crossing each such line with Drosophila flies containing specific genetic make-up, wherein during each such cross the transposable element is capable of moving the reporter gene to a new site in the genome; and

screening the progeny of such cross for alteration in reporter gene activity in the mushroom bodies.

23. The protein sequence identified in SEQ ID NO. 1.

24. The protein sequence of claim 23 in which the first 63 amino acids are replaced with the 63 amino acids identified in SEQ ID NO. 2.

25. The method of claim 2, wherein the protein sequence is SEQ ID NO.1 or SEQ ID NO. 1 wherein the first 63 amino acids are replaced by SEQ ID NO. 2.

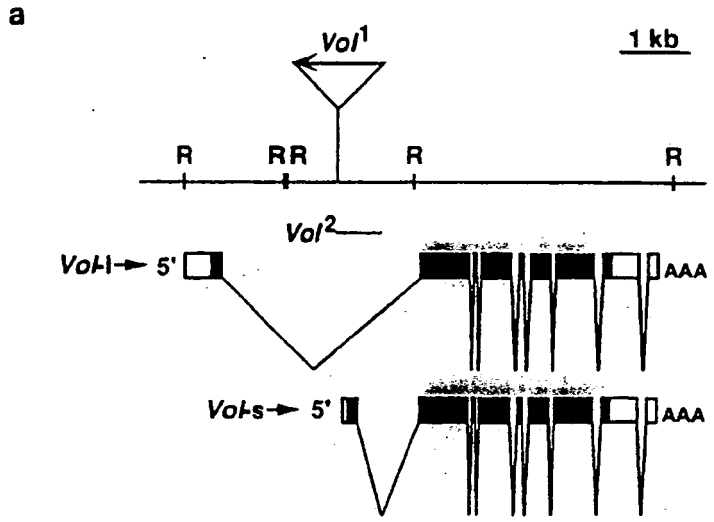
a
Vol1
1 kb
R
RR
R
R
Vol2
Vol-l
5'
AAA
Vol-s
5'
AAA

FIG. 1A

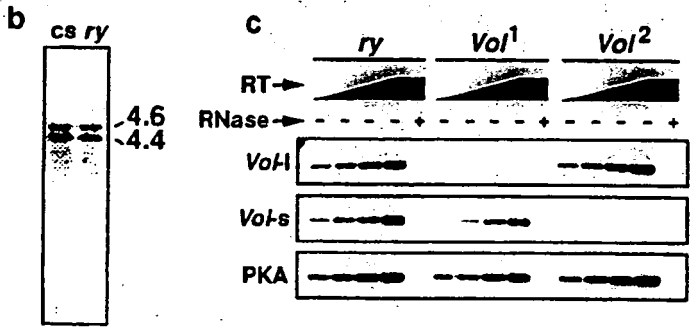
b
cs ry
4.6
4.4
c
ry
Vol1
Vol2
RT
RNase
Vol-l
Vol-s
PKA

FIG. 1B

FIG. 2A

FIG. 2B

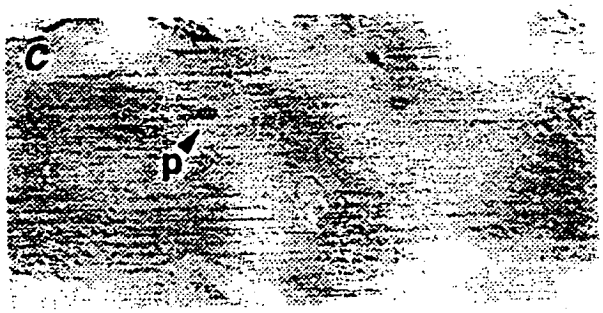

FIG. 2C

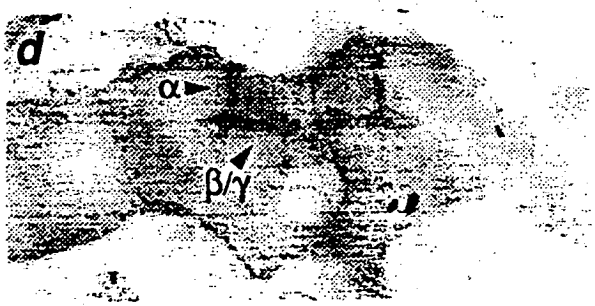

FIG. 2D

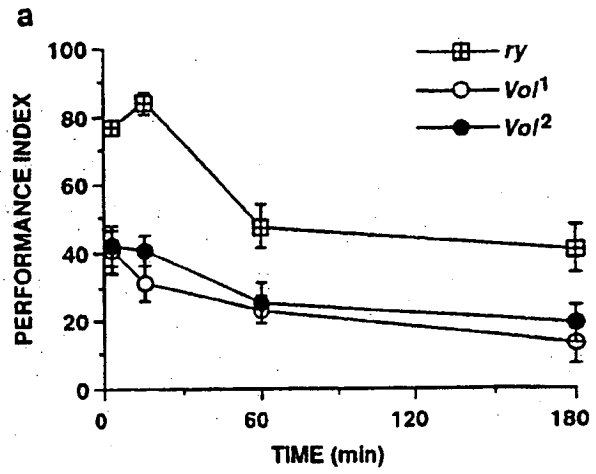
a
PERFORMANCE INDEX
100
80
60
40
20
0
0
60
120
180
TIME (min)
ry
Vol1
Vol2

FIG. 3A

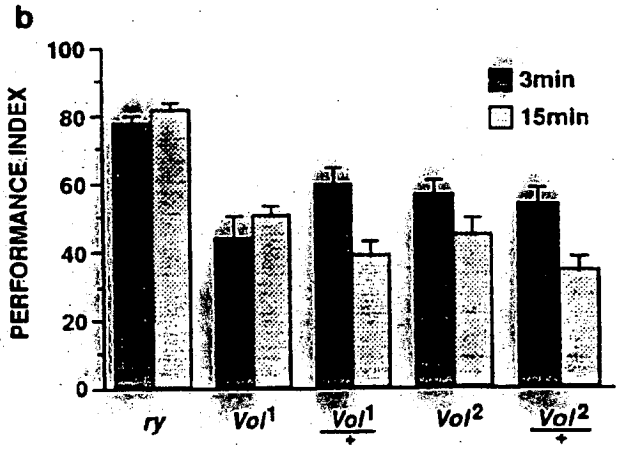
b
PERFORMANCE INDEX
100
80
60
40
20
0
3min
15min
ry
Vol1
Vol1
+
Vol2
Vol2
+

FIG. 3B

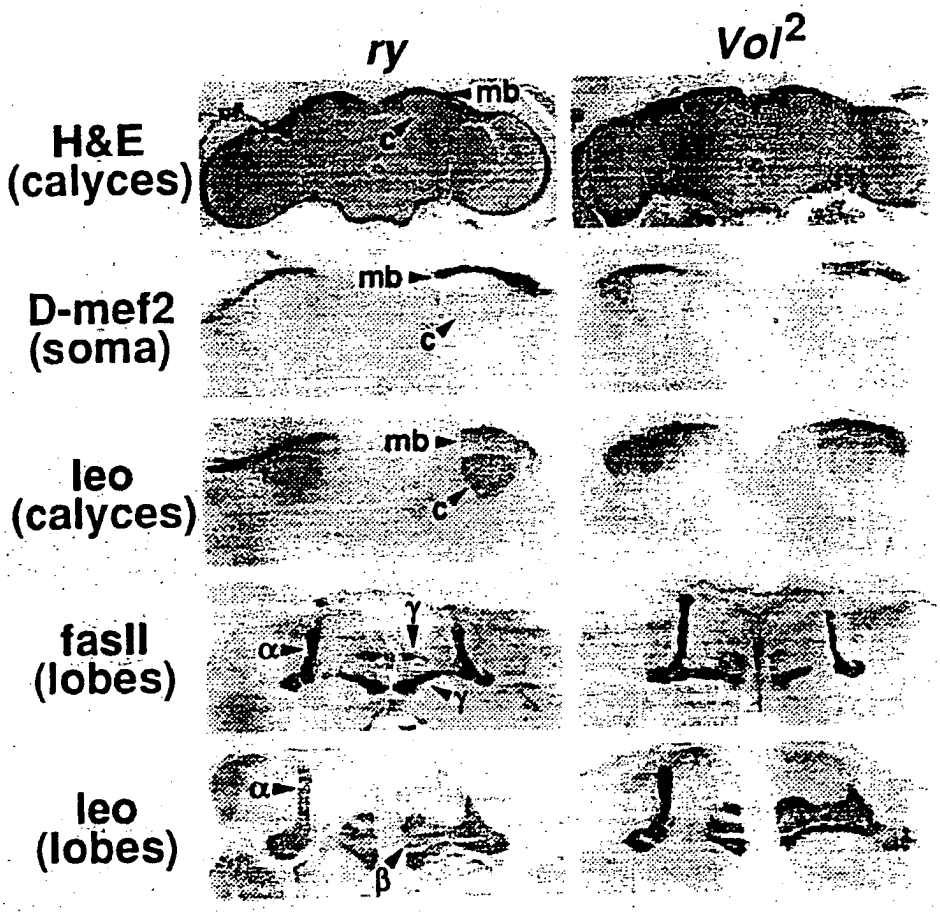
ry
Vol2
H&E (calyces)
D-mef2 (soma)
leo (calyces)
fasII (lobes)
leo (lobes)
mb
c
α
β
γ

FIG. 4

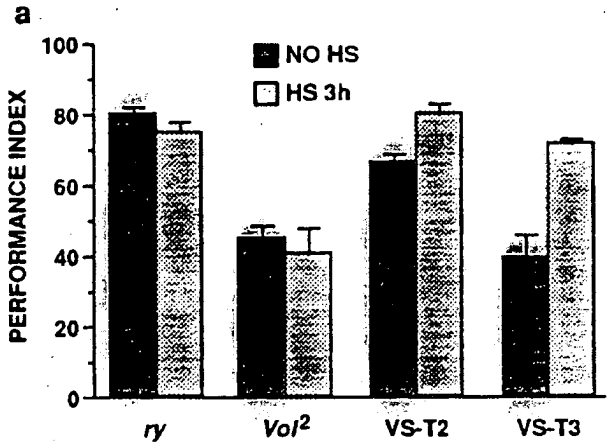
a
NO HS
HS 3h
PERFORMANCE INDEX
100
80
60
40
20
0
ry
Vol2
VS-T2
VS-T3

FIG. 5A

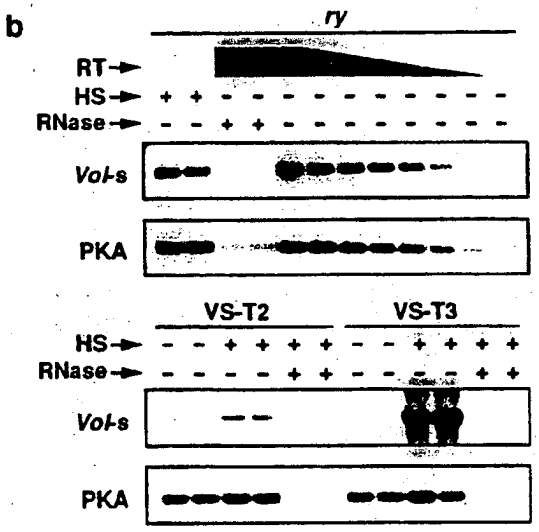
b
ry
RT
HS
RNase
Vol-s
PKA
VS-T2
VS-T3
HS
RNase
Vol-s
PKA

FIG. 5B

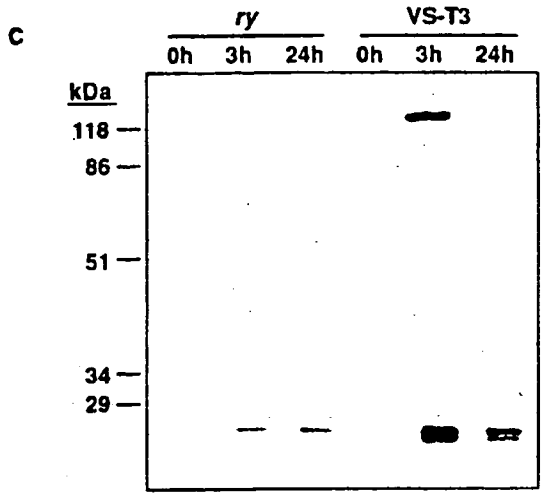

FIG. 5C

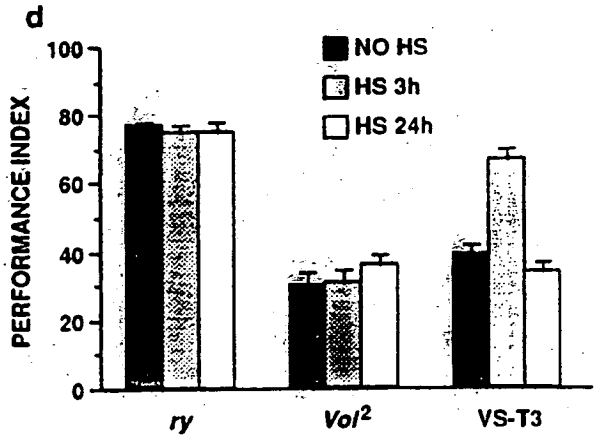

FIG. 5D

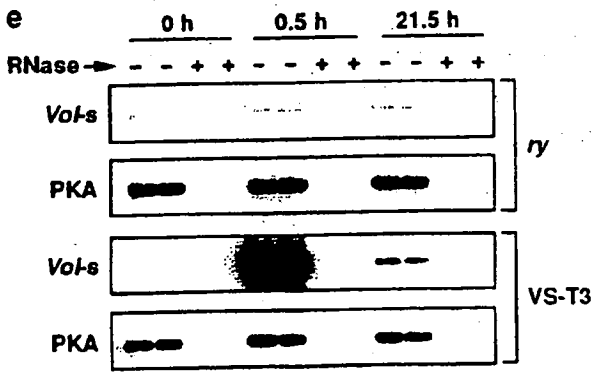

FIG. 5E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01592

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/00, 16/00; C12N 15/00; C12Q 1/00; G01N 33/53
US CL :435/4, 7.1; 530/350, 387.1; 800/3, 13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 7.1; 530/350, 387.1; 800/3, 13

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SKOULAKIS et al. Olfactory learning deficits in mutants for leonardo, a Drosophila gene encoding a 14-3-3 protein. Neuron. November 1996, Vol. 17, pages 931-944, entire document.	1-25
Y	BEHAN et al. Displacement of corticotropin releasing factor from its binding protein as a possible treatment for Alzheimer's disease. Nature. 16 November 1995, Vol. 378, pages 284-287, entire document.	1-25
Y	ZHU et al. Volado: A gene encoding a novel α-subunit of integrin which influences learning. Abstracts of Papers Presented at the Meeting on Neurobiology of Drosophila. 1995, page 9.	1-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 APRIL 1999	13 MAY 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
ANNE-MARIE BAKER, PH.D.
Telephone No. (703) 306-0196

JOYCE BRIDGERS
PARALEGAL SPECIALIST
CHEMICAL MATRIX

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01592

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HEINRICHS et al. Enhancement of performance in multiple learning tasks by corticotropin-releasing factor-binding protein ligand inhibitors. Peptides. 1997, Vol. 18, No. 5, pages 711-716.	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01592

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS
Dialog (file: medicine)
search terms: volado, drosophila, learning, memory, cognit?, integrin, spodoptera